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PRINCIPAL INVESTIGATOR: Sandra L. Grimm, Ph.D.
Jeffrey M. Rosen, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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6. AUTHOR(S)
Sandra L. Grimm, Ph.D.
Jeffrey M. Rosen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030

E-Mail: sgrimm@bcm.tmc.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Targeted deletion of the bZIP transcription factor, C/EBPß, was shown previously to result in aberrant ductal morphogenesis and decreased lobuloalveolar development, accompanied by an altered pattern of progesterone receptor (PR) expression. Similar changes in the level and pattern of prolactin receptor (PrlR) expression were observed while screening for differentially expressed genes in C/EBPß KO mice. PR patterning was also altered in PrlR KO mice, as well as in mammary tissue transplants from both PrlR KO and Stat5a/b-deficient mice, with concomitant defects in hormone-induced proliferation. Downregulation of PR and activation of Stat5 phosphorylation were observed following estrogen and progesterone treatment in both C/EBPß KO and wildtype mice indicating that these signaling pathways were functional, despite the failure of steroid hormones to induce proliferation. IGFBP-5, IGF-II, and IRS-1 all displayed altered patterns and levels of expression in C/EBPß KO mice, suggestive of a change in the IGF signaling axis. In addition, SPRR2A, a marker of epidermal differentiation, and keratin 6 were misexpressed in the mammary epithelium of C/EBPß KO mice. Together, these data suggest that C/EBPß is a master regulator of mammary epithelial cell fate and that the correct spatial pattern of PR and PrlR expression is a critical determinant of hormone-regulated cell proliferation.

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• Introduction

The study of mouse knockout (KO) models has helped elucidate the *in vivo* function of many different genes involved in the various stages of mammary gland development. Previous analyses of C/EBPß KO mice have demonstrated an important role for this transcription factor in ductal morphogenesis and lobouloalveolar development of the mammary gland, and suggested that detailed molecular studies of this mouse model might provide new insights into the mechanisms responsible for these processes. The correlation of increased PR expression coupled with an inhibition of proliferation provides a novel way of viewing PR signaling events. The identification of genes and signaling pathways involved in the normal mammary gland development may provide novel targets for the treatment of breast cancer.

• Body

Objective 1: *Investigate the potential role of known genes that may mediate the paracrine action of PR required for alveolar proliferation using the C/EBPß-/- mouse model.*

Task 1A
- *Isolate total RNA from virgin and E+P treated mammary glands from C/EBPß wildtype and knockout mice*
- *Construct riboprobe vectors*
- *Perform RPA and Northern analyses*

I will continue to collect mammary gland tissues from both wildtype and C/EBPß KO mice. I will also be collecting embryos between 12.5 to 18.5 days to analyze the expression of potential mammary progenitor cell markers in the developing mammary buds (part of Task 1C). Mammary glands from 1, 2, or 3 week old mice will also be collected to prepare paraffin-embedded sections for immunohistochemical studies in Task 1C.

Based on our previous observation that IGFBP-5 decreases in the mammary glands from C/EBPß KO mice, we were interested in looking at other factors in the IGF signaling axis. Insulin receptor substrate (IRS) proteins are cytoplasmic molecules that transmit signals from a number of growth factor receptors, including insulin receptor and IGF-IR. Two forms are expressed in the mammary gland: IRS-1 and IRS-2. Riboprobe vectors for these genes were obtained from Darrell Hadsell at Baylor College of Medicine and used in RPA analyses. I found no change in the mRNA levels of IRS-1 or IRS-2 between wildtype and C/EBPß KO mice.

Future RPA analyses will make use of multiprobe template kits available from Pharmingen (San Diego, CA). Because of the decrease in proliferation in the C/EBPß KO mammary glands, candidate genes include cyclins (mCYC-1 and mCYC-2 probe sets) and other cell cycle components (mCC-1 probe set).

Task 1B • *Perform* in situ *hybridization on paraffin-embedded mammary gland sections, comparing WT and KO, using riboprobes from above*

Continuing our collaboration with Terri Wood at Penn State, we have performed *in situ* hybridization for IGF-II on frozen sections. When expression was analyzed at 6 weeks, levels of IGF-II were similar between WT and C/EBPß KO and mRNA was uniformly expressed along the ducts. By 12 weeks, IGF-II mRNA was decreased in the WT and a punctate pattern of expression was observed. However, IGF-II was increased and uniform along the ducts from 12-week C/EBPß mice. This expression pattern was reminiscent of that seen for PR and prolactin receptor. Normally, expression of these genes is downregulated between 6 and 12 weeks due to the increase in circulating ovarian hormones during this time frame. In the C/EBPß KO, the expression of these genes/proteins is never downregulated and remains elevated.

The bulk of visualizing gene expression in the mammary gland will be performed by immunohistochemistry, as described in Task 1C. A major advantage to the immunostaining experiments is the ability to colocalize protein expression by using immunofluorescence. However, for certain secreted growth factors, or when suitable antibodies are unavailable, *in situ* hybridization will be performed.

Task 1C • *Obtain antibodies to candidate proteins and perform immunohistochemistry.*

Although there was no difference in the level of IRS-1 mRNA, Western blot analysis showed a decrease in protein levels in the C/EBPß KO. Using antibodies against IRS-1, immunohistochemistry on WT and C/EBPß KO sections revealed a decrease in protein expression in the KO glands. In addition to the overall levels decreasing, a change in the expression pattern was also observed. Similar to the pattern of IGFBP-5, IRS-1 expression went from uniform expression in the WT to punctate, non-uniform expression in the C/EBPß KO. This change in patterning correlates with a 10-fold decrease in proliferation, suggesting these IGF axis signaling molecules may be involved in the proliferative response. However, when double immunofluorescence was performed with IRS-1 and BrdU antibodies, the two antigens did not always colocalize to the same cells. The BrdU-positive cells often expressed IRS-1, but not all IRS-1-positive cells were proliferating. Immunoprecipitation of IRS-1 from whole cell extracts and subsequent Western blotting with an anti-phospho tyrosine antibody showed no difference in tyrosine phosphorylation between WT and KO. So it appears that the expression levels and patterning of IRS-1 change in the C/EBPß KO, rather than an alteration in protein phosphorylation.

While the levels of PR and PrlR increased in the mammary glands of C/EBPß KO mice, it is not clear if the signaling pathways in these cells are functional. Turnover and downregulation of PR protein results from prolonged exposure to P. Therefore, wildtype and C/EBPß KO mice were implanted with E+P pellets for 21 days to determine if PR levels could be downregulated after chronic exposure to steroid hormones. Comparing the percentage of PR-positive MECs from untreated animals with those treated for 21 days with E+P showed a 2-fold decrease in the glands from wildtype mice. Although the level of PR was

almost three times greater in C/EBPß KO mice prior to treatment, the fold-downregulation after hormone treatment was identical.

The ability of Prl acting through the PrlR to activate the Jak/Stat pathway, leading to the tyrosine phosphorylation of Stat5, was also determined by immunoprecipitation-Western blot analysis of whole cell mammary gland extracts. Levels of Stat5 tyrosine phosphorylation were similar in extracts from glands from either untreated or hormone-treated wildtype mice. Interestingly, no Stat5 tyrosine phosphorylation was detected in mammary gland extracts from untreated C/EBPß KO mice, but tyrosine phosphorylation was observed after acute hormone treatment. There were no significant differences in serum Prl levels between the untreated animals of either genotype and there was an approximate 50% increase after 2 days of E+P treatment for both genotypes.

Surprisingly, another gene identified by an SSH-PCR screen (Task 2A) was the small proline-rich protein 2A (SPRR2A), a marker of epidermal differentiation that is normally expressed in the cornified layer of the skin (1). Previous Northern blot analysis confirmed the differential expression of SPRR2A and demonstrated that the level of SPRR2A mRNA was markedly increased in the mammary glands from nulliparous C/EBPß KO mice. Immunohistochemistry was performed to determine the levels and pattern of SPRR2 protein expression in the mammary gland. There was no staining in wildtype glands but a punctate, non-uniform pattern of expression was observed in the MECs from C/EBPß KO glands, similar to the pattern seen for PR and PrlR. Similar results were seen in tissues from both untreated animals and those treated for 2 days with E+P.

This finding led us to investigate whether other epidermal markers, such as keratins, were expressed in the mammary glands of C/EBPß KO mice. Keratin 10 staining was negative in both wildtype and C/EBPß KO glands, but was positive on a section of newborn skin. Keratin 6 (K6) is involved in wound healing and is associated with hyperproliferative diseases (2). While K6 is expressed in the body cells of the TEBs in the pubertal mammary gland, it is rarely detected in ducts of the mature gland (3, 4). Consistent with these findings, we saw no K6 immunoreactivity in wildtype MECs. However, K6-positive MECs were readily apparent in ducts from mature C/EBPß KO mice. While the immunoreactivity was more uniform than that observed for SPRR2A, not all MECs were K6-positive. Keratin 14 expression is normally detected in the myoepithelial cells surrounding the ducts, and no differences were detected in the staining patterns in the myoepithelium between wildtype and KO mice.

Another gene identified by the SSH-PCR screen in Task 2A is the sodium-potassium-chloride cotransporter NKCC1. Expression of this protein has been characterized as a marker of ductal epithelial cells, and is not expressed in funtionally-differentiated alveolar cells (5). Immunofluorescence using an NKCC1 antibody from Jim Turner (National Institute of Craniofacial and Dental Research, NIH, Bethesda, MD) shows expression of NKCC1 is upregulated in the C/EBPß KO gland. Again, the expression pattern shifts from non-uniform in WT mice to uniform in C/EBPß KO mice. Similar to PR, NKCC1 expression appears to be dissociated from proliferation.

The model we have proposed suggests that the PR-positive cells, which do not proliferate, express the cyclin-dependent kinase inhibitor p27. Immunohistochemistry for p27 on WT and C/EBPß KO mammary gland sections

shows an increase in the number of cells expressing p27. When quantitated, the WT glands have 23% of MECs expressing p27, whereas C/EBPß KO ducts have 42% p27-positive cells. The increase in p27 expression correlates with a 10-fold decrease in proliferation in C/EBPß KO mice.

It appears likely that the expression of these markers may represent a block in the normal cell fate determination and development pathway as a consequence of the germline deletion of C/EBPß. To further investigate this possibility, I will be isolating both wildtype and C/EBPß KO embryos at various timepoints (E12.5 through E18.5) to analyze the expression of various proteins in the developing mammary placodes. I will also isolate mammary glands between 1 and 3 weeks of age to determine the expression of the same markers in the terminal end buds (TEBs). Some of the proteins that will be analyzed by immunohistochemistry or immunofluorescence include keratin 6, keratin 14, keratin 5, p63, Lef-1, C/EBPß, PHTrP, Sca-1 (Ly-6A/E), SPRR2A, NKCC1, estrogen receptor, progesterone receptor, androgen receptor, E-cadherin, and tenascin C. This should provide both a comprehensive survey of the spatial and temporal expression of these proteins in the wildtype embryo, as well as exploiting C/EBPß KO mice as a tool to identify early changes in mammary gland progenitor cell markers. Optimization for approximately half of these antibodies has been completed.

Task 1D • *Implant hormone-containing pellets in mammary glands of C/EBPß -/- mice.*

Not addressed at this time.

Task 1E • *Mix C/EBPß -/- and ROSA26 mammary epithelium, transplant into RAG1 -/- females. Impregnate engrafted mice and harvest mammary glands at timepoints during pregnancy.*

This aim was proposed to verify the hypothesis of a paracrine/juxtacrine mechanism of action where PR-positive cells influence the neighboring cells to induce proliferation, and similar experiments using PRKO, PrlR KO and Wnt-4 KO cells have been performed (6-8). Considering the technical difficulties of this technique, the number of mice required, and the discoveries we have made in identifying molecular changes in the C/EBPß KO mammary gland, we no longer feel this task should be pursued. In light of our data supporting the hypothesis of a block in the progenitor cell lineage, we will instead be focusing on earlier developmental events during embryonic mammary bud formation and ductal elongation during the first three weeks of mammary gland development, as described above.

Objective 2: *Identify and characterize novel downstream gene targets differentially expressed in the C/EBPß-/- mammary gland.*

Task 2A • *Screen KO tester and WT tester libraries for additional differentially expressed partial cDNA clones*

• *Confirm change in gene expression of SSH-PCR clones by Northern*
• *Construct normalized midpregnant mouse mammary gland cDNA library [Revised to virgin mammary gland cDNA library]*

With the sequencing of the mouse and human genomes, many of the approximately 60 genes identified in the SSH-PCR screen (9) have now been identified. Examples of genes identified by this screen that have been investigated further are SPRR2A, IGFBP-5 and NKCC1. A few ESTs were also identified, which will continue to be checked against the GenBank databases to identify the genes they represent. If they remain unknown genes, the full length cDNAs will be isolated from the mature virgin cDNA library already constructed and described in my last report.

Task 2B • *Screen cDNA library for full length cDNA clones*
• *Sequence analysis of full length cDNAs*

In my last report, I described the cloning of a partial cDNA representing an unknown gene identified by the SSH-PCR screen in Task 2A that is upregulated in the C/EBPß KO. The clone, called F28, was most closely related to a gene found in many species, including CRP-ductin in mouse (10), hensin in rabbit (11), DMBT1 in human (12), and ebnerin in rat (13). With the sequence of the mouse and human genomes available, a BLAST search using the 4.3 Kb of F28 sequence identified the genomic mouse sequence that matched the partial cDNA. The F28 gene is located on mouse chromosome 7 (band F4) and the human F28 gene is found on the syntenic region of human chromosome 10 (q25-26). Analysis of the genomic sequence using Genescan (http://genes.mit.edu/GENSCAN.html) predicts approximately 55 exons spanning 135 Kb, placing F28 50 Kb downstream of the CRP-ductin gene. The partial cDNA was aligned to the genomic sequence using the SIM4 program (http://biom3.univ-lyon1.fr/sim4.html) to test the validity of the cDNA prediction with Genescan. The predicted cDNA of 8610 bp encodes an open reading frame (ORF) of 2699 amino acids (aa). Predominant characteristics of the predicted protein include a good Kozak consensus sequence surrounding the translation start site (14), a signal peptide at amino acid 24, 4.7% cysteine residues, and 17.6% serine/threonine residues that have the potential to be glycosylated. The Ser/Thr-rich region is concentrated to approximately 400 aa at the C-terminus. Domain mapping using SMART (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de) identified twelve SRCR (scavenger receptor cysteine rich) domains (15), four CUB domains (16), one ZP (Zona pellucida) domain (17), but no transmembrane domain at the C-terminus. The conservation of the domain content and order between F28 and CRP-ductin/DMBT1/hensin implies that F28 will have a similar function to these known proteins.

There was also 65% identity between first 500 bp of the 5' UTR of the mouse and human sequences, suggesting the promoter regions are conserved. Mapping of putative transcription factor binding sites in the 5' UTR is in progress.

Task 2C • *Preliminary gene characterization*

Not addressed at this time.

• Key Research Accomplishments

• Altered expression and localization of IGF axis molecules (IGF-II, IRS-1) in mammary glands of C/EBPß KO mice.

• Hormonally-regulated signaling pathways in the mammary glands of C/EBPß KO mice are functional as shown by hormone-induced downregulation of PR and phosphorylation of Stat5.

• Increased expression of NKCC1, a marker of ductal epithelium, in C/EBPß KO mammary glands.

• Increased expression of p27, a cell-cycle inhibitor, in C/EBPß KO mammary glands correlates with a 10-fold decrease in proliferation.

• SPRR2A, a marker of epidermal differentiation, and keratin 6 are inappropriately expressed in the mammary glands of C/EBPß KO mice, as determined by immunohistochemistry.

• Identification of a novel gene (designated F28) that is related to CRP-ductin/DMBT/hensin. Domain mapping and exon boundary mapping are complete for genomic sequence, predicted cDNA and putative ORF.

•Reportable Outcomes

Presentations/Abstracts:
• Gordon Conference on Mammary Gland Biology
Barga, Italy (April 28 – May 3, 2002)
Poster title: Disruption of steroid receptor patterning correlates with a block in lobuloalveolar development in multiple mouse knockout models

Manuscripts:
• Signal Transducer and Activator Transcription 5 (Stat5) controls the specification of mammary alveolar epithelium.
Keiko Miyoshi, Jonathan Shillingford, Gilbert H. Smith, Sandra L. Grimm, Kay-Uwe Wagner, Takami Oka, Jeffrey M. Rosen, Gertraud W. Robinson and Lothar Hennighausen. 2001.
Journal of Cell Biology **155:** 531-542.

• Jak2 is an essential tyrosine kinase involved in pregnancy-mediated development of mammary secretory epithelium.
Jonathan Shillingford, Keiko Miyoshi, Gertraud W. Robinson, Sandra L. Grimm, Jeffrey M. Rosen, Hans Neubauer, Klaus Pfeffer, Lothar Hennighausen. 2002.
Molecular Endocrinology **16:** 563-570.

• **Disruption of steroid receptor patterning correlates with a block in lobuloalveolar development in multiple mouse knockout models.**
Sandra L. Grimm, Tiffany N. Seagroves, Elena B. Kabotyanski, Russell C. Hovey, Barbara K. Vonderhaar, John P. Lydon, Keiko Miyoshi, Lothar Hennighausen, Christopher J. Ormandy, Adrian V. Lee, Malinda A. Stull, Teresa L. Wood, Jeffrey M. Rosen
Submitted to Molecular Endocrinology

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Competing renewal of NIH grant CA16303 MERIT award to Jeffrey Rosen.

•Conclusions

By analyzing the very earliest events in mammary gland development, I hope to be able to pinpoint changes in gene expression that are responsible for, or markers of, the lack of lobuloalveolar development in the C/EBPß KO mice. So far, we have observed changes in the expression of a number of genes that are known to influence mammary gland development. However, the genes identified from SSH analysis may provide the greatest insight into this process. Preliminary results indicate that it may not be a block in lobuloalveolar development, per se, but an alteration in cell fate at an earlier stage that may be responsible for the phenotype observed. Support for this hypothesis is the observation of overexpression of SPRR2A, a marker of skin differentiation, and keratin 6 in the C/EBPß KO mammary gland. The approach we are taking should be able to identify genes and signaling pathways involved in the normal development of the mammary gland, which may provide novel targets for the treatment of breast cancer.

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